SF 298 MASTER COPY KEEP THIS COPY FOR REPRODUCTION PURPOSES

REPORT DOCUMENTATION PAGE

Form Approved OMB NO. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	1/30/96	Final Report: 10/1/95 - 12/31/95

4. TITLE AND SUBTITLE

Development and Application of Low-Temperature AFM

5. FUNDING NUMBERS

DAAH04-95-1-0633

6. AUTHOR(S)

Jie Yang

7. PERFORMING ORGANIZATION NAMES(S) AND ADDRESS(ES)

Physics Department
University of Vermont
Cook Building
Burlington, VT 05405

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Research Office
P.O. Box 12211
Research Triangle Park, NC 27709-2211

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

ARO 34147.1-LS

11. SUPPLEMENTARY NOTES

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision, unless so designated by other documentation.

12a. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release; distribution unlimited.

12 b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

With the support from the US Army Research Office, we have continued our efforts in biological applications of atomic force microscopy. Our instrumentation development, based on what we have achieved with a prototype low temperature atomic force microscope, will allow us to conduct structural studies under a controlled temperature over a wide range. We have developed various methods to facilitate atomic force microscopy of biological specimens at room temperature. Preparing supported bilayers made of cationic lipids enabled us to image DNA molecules in solution at a resolution high enough to resolve the pitch of DNA. We also discovered that membrane-bound DNA molecules are closely packed. Using mica chips as substrates allows us to study thermal properties of supported bilayers with a differential scanning calorimeter. Our results show that there is a new high-temperature phase in supported phospholipid membranes. We have recently developed a method to use a protein label to study the process of how a hydrophobic entity inserts into a supported bilayer. This will help us to understand the insertion process which is essential for the understanding of the function of many integral membrane proteins.

14. SUBJECT TERMS

15. NUMBER IF PAGES
9

16. PRICE CODE

17. SECURITY CLASSIFICATION OR REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
UNCLASSIFIED	UNCLASSIFIED	UNCLASSIFIED	UL

Development and Application of Low-Temperature AFM

Final Report

Jie Yang

January 30, 1996

U.S. Army Research Office

Funding Document DAAH04-95-1-0633

Physics Department
University of Vermont
Cook Building
Burlington, VT 05405

Table of Contents

1. Introduction.

The newly developed atomic force microscope (AFM) has shown its great potential in biological applications. It offers a resolution far beyond what can be achieved with normal light microscopy. Yet, it avoids harsh specimen treatment normally required with electron microscopy. However, the instrument has its own limit in biological applications. One of the most serious problem with AFM of biological specimens is their softness. To overcome this hurdle, we proposed to develop a cryogenic temperature AFM for biological applications. With the support from the US Army Research Office, under the Funding Document DAAL03-92-G-0002, we were able to demonstrate that a properly designed cryo-AFM, operating in nitrogen vapor, could be suitable for bio-imaging at high-resolution, provided the accomplishment of both the construction and the operation of a proper apparatus for freeze-fracturing/freeze-etching of rapidly frozen biological specimens. This establishes a basis for using full potential of the AFM in biological applications. We also have developed substantially both the instrumentation and methodology for room temperature bio-applications of AFM.

With a subsequent support from the US Army Research Office, under the Funding Document DAAH04-95-1-0633, we have been continuing research activities related to the objectives in the original grant (Funding Document DAAL03-92-G-0002) at a new site, University of Vermont. We have widened our horizon in developing methods for structural and functional studies of model membrane, membrane proteins, and DNA molecules. A brief account of our achievement, to which the support from the US Amy Research Office contributes substantially, is given below.

2. Instrumentation.

Our prototype cryo-temperature AFM shows the feasibility of our idea of obtaining a clean environment in cold nitrogen vapor. It also shows that an AFM using optical detection works properly at cryogenic temperatures. Therefore, to achieve our goal of developing a cryo-temperature AFM for biological applications, remaining problems are technical and are soluble on the basis of the present technology. Since we will not require any elaborate high-vacuum apparatus, the construction of parts in our developments should not be too costly.

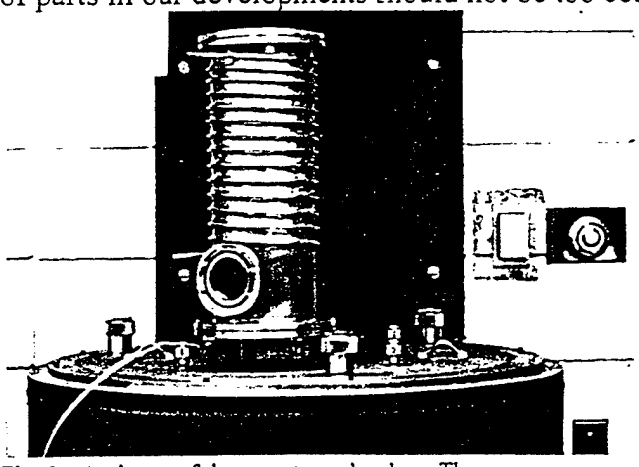

Fig. 1: A picture of the prototype chamber. The copper tubing around the wall is soft soldered on. The chamber is on top of a large cryo-dewar.

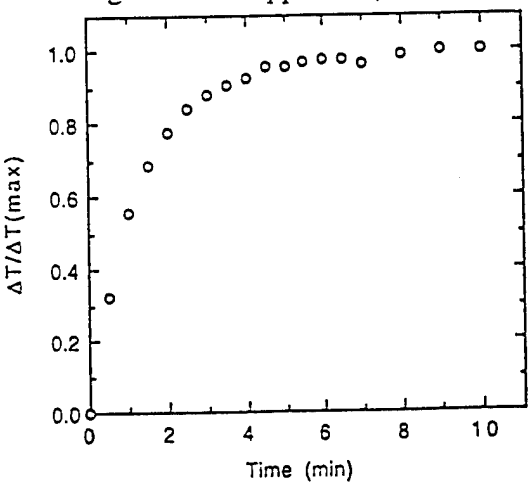

Fig. 2 Relaxation time of the prototype chamber.

One of the most encountered technical problem in normal AFM operation is instrumental drifts caused by temperature instability. This is also one of the problems with our cryo-AFM. Our initial idea of using an AFM chamber circumvents this problem. We further constructed a compact AFM chamber based on our success with the prototype cryo-AFM. It is made of copper, with large top and bottom flanges, to facilitate later incorporation into the more elaborated and thermally insulated chamber as the inner wall. Fig. 1 shows a picture of this prototype chamber. The copper tubing around the wall allows the flow through of cryogen to control the interior temperature. Using hot water (tap water) to warm (cool) the chamber, the thermal equilibrium time of the chamber was determined (Fig. 2). The time constant is 1.5 min. This is sufficiently fast for imaging experiments.

For future applications at temperatures above the freezing point of a solution, this chamber will be used as the inner wall of a double-wall chamber. The two walls will be assembled at the top through a rubber o-ring, to facilitate an easy disassemble. The inside wall will be thermally isolated from the outer wall by vacuum. A view port is installed to facilitate visual guidance of remote operations, such as the adjustment of laser orientation or photodetector position. The temperature will be regulated by connecting the copper tubing to a temperature regulated refrigerating circulator. Inside the chamber, a fan driven by a motor will shorten the thermal equilibrium time. A baffle assembly is attached to the top flange of the chamber to isolate the imaging environment thermally. Shafts, that can grab the screws for photodetector-positioning and for laser beam-orienting, are sealed at the top flange by rubber o-rings.

3. *Applications.*

Our constant developmental work has enabled us to apply the novel technology to study various biological systems. In a short period since I have moved to the University of Vermont, we were able to establish several new fronts in biological AFM. Following are some details.

3.a. Studies of membrane-bound DNA

Our success with AFM of membrane-bound cholera toxins indicates that a resolution of close to 1 nm can be obtained with *in situ* AFM for properly prepared specimens. It certainly will be helpful to realize high-resolution structural studies of DNA in solution with an AFM. For imaging in air or under an organic solvent, the resolution, obtained with an AFM on DNA molecules, is not any better than that with an electron microscope (EM). This is not sufficient to resolve any structural details beyond the helical pitch. We have come to the conclusion that the main problem lies on specimen preparation. On the basis of our success with various methods of preparing supported bilayers, we have developed a method to prepare cationic supported bilayers as substrates for tethering DNA to the membrane.

The use of supported cationic bilayers enabled us to image membrane-bound DNA molecules in solution at a resolution high enough to resolve the helical repeat. Our mean value of the pitch of DNA is consistent with the helical periodicity of DNA determined by enzyme digestion. A wide distribution of the pitch is found, with the pitch of 3.5 ± 0.6 nm for a plasmid and 3.6 ± 0.6 nm for DNA fragments. We also examined the physical mechanism of the present method, and obtained a lower limit for the binding strength of DNA to the cationic lipid membrane to be about 5 kcal/mol per helical turn. Besides a strong binding of DNA to the substrate, a surprising strong attraction of DNA on the

substrate was found. This causes a close-packing of membrane-bound DNA molecules. We also examined various specimens and investigated the physical characteristics of the method to eliminate any possibility of artifacts. Fig. 3 shows two examples of membrane bound plasmid DNAs and DNA fragments.

Fig. 3a: Here is a typical image of membrane-bound plasmid DNA in solution. Notice the close packing, and the high resolution feature directly observed in this image. The pitch of DNA can be seen on many segments along the DNA molecule.

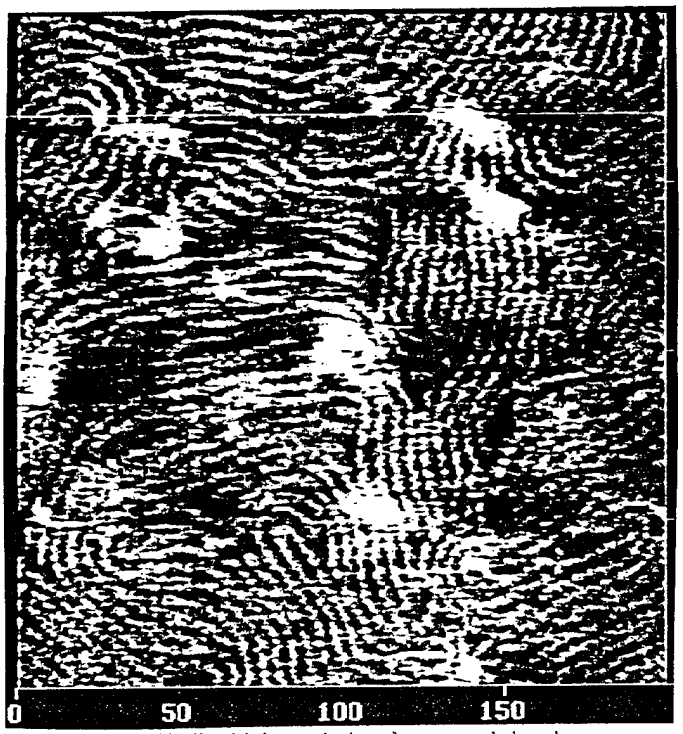

Fig. 3b: Similar high resolution feature and the close-packing are seen on DNA fragments, as shown here. The distribution of the pitch of DNA in this case is not any narrower than that of the plasmids.

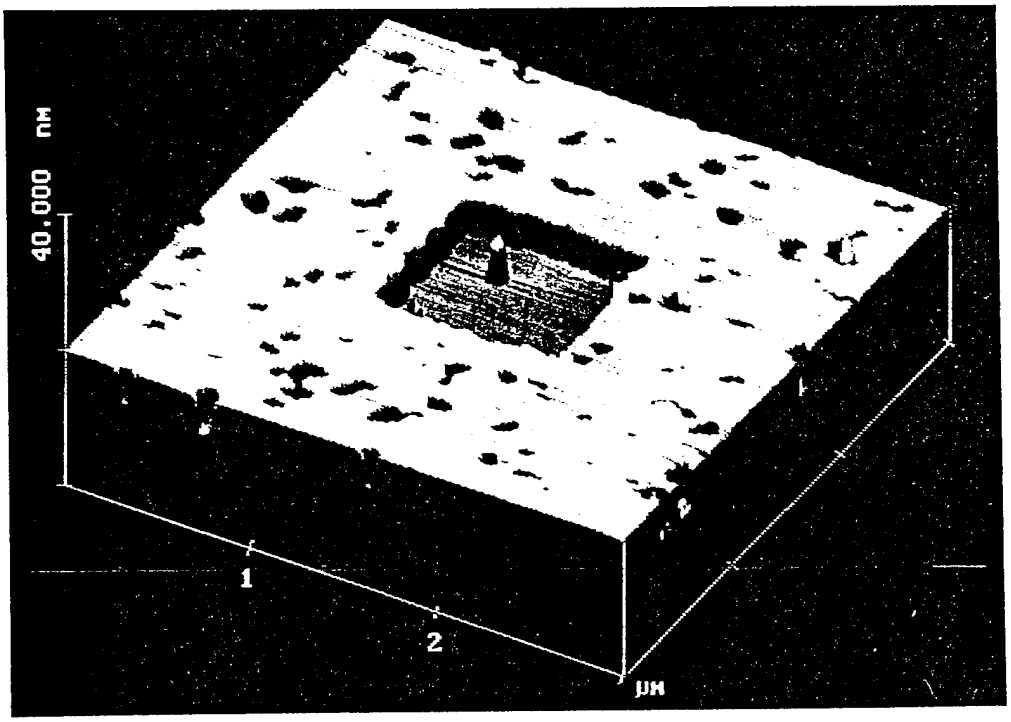

Fig. 4: DPPC bilayer prepared by the vesicle-fusion method on a mica surface. The dark areas are bilayer defects. The thickness of the bilayer (~ 6 nm) was determined at the edges of these defects.

3.b. Structural studies of model membranes

Since cell surfaces proved too soft for *in situ* high-resolution imaging of membrane proteins, supported membranes have become essential substrates for structural determination of membrane proteins by AFM. Over the past three years, we have successfully developed several methods for preparing supported bilayer membranes. Using the Langmuir trough, supported bilayers were obtained by transfer of two monolayers of lipid molecules at an air-water interface onto a mica surface *via* two strokes of a vertical dipper. This enabled us to obtain supported bilayers of gel-phase phospholipids, such as dipalmitoyl-, distearoyl-, and dipentadecanoylphosphatidylcholine (DPPC, DSPC and diC15-PC). We also established a method to prepare supported bilayers of gel-phase lipids by fusing lipid vesicles on a substrate. Supported bilayers of similar quality were prepared with this method without any difficulty. Figure 4 shows an example of DPPC bilayer prepared by the vesicle-fusion method.

Our methods of preparing supported bilayers have established a basis for structural studies of membrane proteins. One goal for these studies is to know the feasibility of incorporation of membrane proteins into supported bilayers. In a first step toward this goal, we tested the possibility of incorporating a membrane receptor, the ganglioside G_{M1}, into supported bilayers. It was found that gangliosides incorporated into supported bilayers of different lipids in their fluid phase. The evidence of sufficient incorporation was obtained by imaging membrane-bound cholera toxin B-oligomers (CTBs), since they bind to the ganglioside G_{M1} with unusually high affinity ($K_d \sim 10^{-9}$ Mole). The structure of membrane-bound CTBs is obtained at a resolution about 1 nm, sufficient to resolve the central pore and some details of subunit arrangement. From the example shown in Fig. 5, it is seen that some of the molecules orient at different orientation on the bilayer, rather than sitting down flatly on the bilayer plane. Some of the oligomers show nonsymmetric arrangement of their subunit, although many of them show the symmetric pentameric structure. Although preliminary, we show convincingly that the incorporation of membrane proteins should be possible if hydrophobic interaction is the main driving force, since the incorporation of the ganglioside is due to the hydrophobic interaction. For integral membrane proteins, the magnitude of this hydrophobic interaction may be weaker. Therefore, a longer time and a higher temperature may be needed for the incubation.

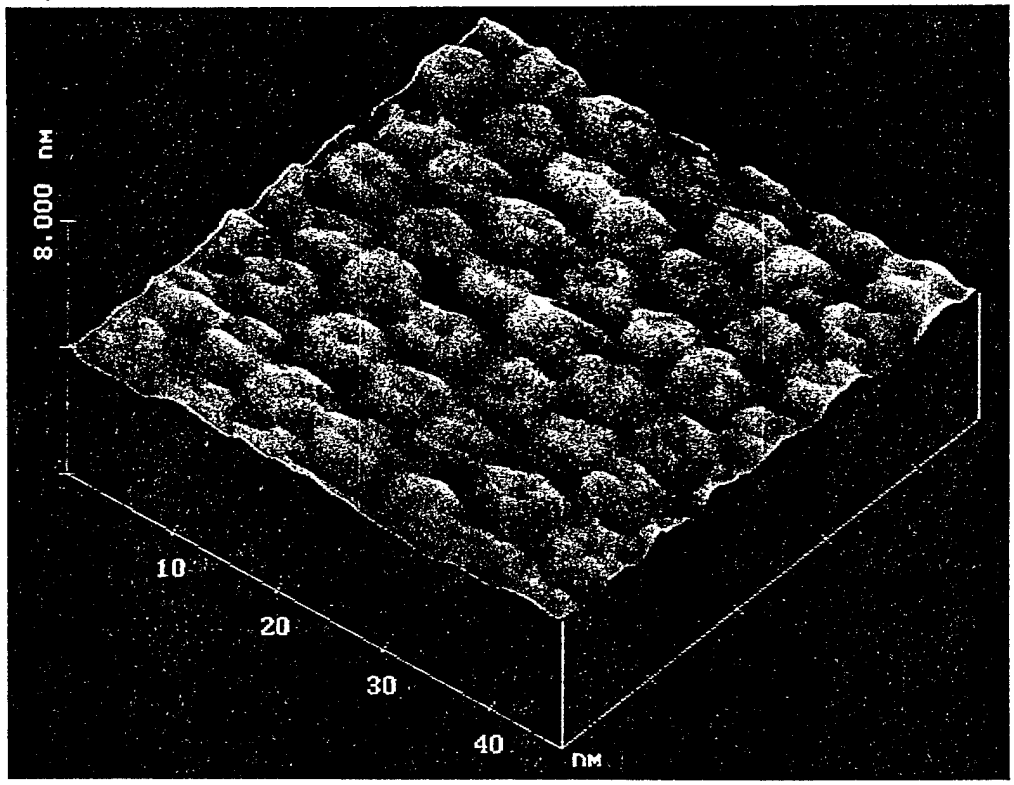

Fig. 5: Randomly distributed membrane-bound cholera toxin B-oligomers on a supported egg-PC bilayer with the receptor incorporated by direct incubation.

3.c. Thermoentropic studies of supported bilayers

Studies of thermal properties of supported bilayers are of great importance, since they are essential for understanding the structure of model membranes and will provide useful information to incorporate membrane proteins into supported bilayers. However, under normal conditions, the substrate area of a supported bilayer is much smaller than what is required for thermal studies of large ensembles. To overcome this shortcoming, I developed a method by using mica chips as substrates for supported bilayers. This increases significantly the substrate area. Therefore, thermal properties of supported bilayers can be studies with a differential scanning calorimeter (DSC).

I examined the thermodynamics of supported bilayers, and found a new high-temperature phase in supported bilayers, although the structure of supported bilayers is unusually stable. I also found the existence of extremely slow dynamic phase behaviors of lipid bilayers. Fig. 6a shows typical curves from a DSC study of supported DPPC bilayer. Fig. 6b shows the variation of the main phase transition temperature (T_m) and the high-temperature phase transition temperature (T_h) as a function of time. Fig. 6c shows that the total transition enthalpy (ΔH), the main phase transition enthalpy (ΔH_m), and the ratio of ΔH_m over the high-temperature transition enthalpy (ΔH_{HT}) remain a constant within experimental error. These show that we can prepare routinely supported bilayers, can raise the temperature of the bilayer for membrane-protein incorporation or for 2-D crystallization, and can then to lower the temperature, if necessary, for stable imaging. The results of our DSC studies are interesting in their own sake, and show the capability in this lab to conduct thermal studies of other system of interest in the near future.

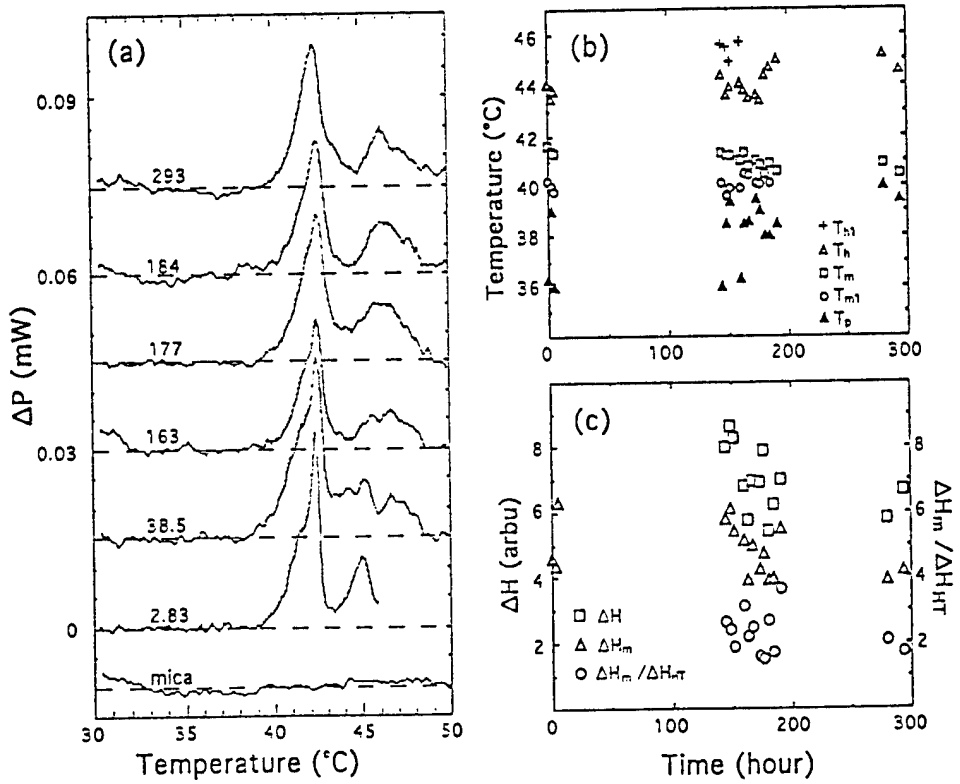

Fig. 6: (a) shows a set of DSC scanning curves of mica supported DPPC bilayers. The high temperature peak after the main transition shows a new high temperature phase in supported bilayers. The shape change of the transition peaks indicates the dynamic phase behavior of supported bilayers. (b) and (c) show the transition temperatures and the Transition enthalpies as a function of time. It shows a slow variation of the transition temperatures (T_m and T_h). However, the transition enthalpies remain a constant within experimental error.

4. Publications.

Abstracts:

1. The physical mechanism of the close-packing of membrane-bound DNA in solution.
   Jie Yang, Lijiang Wang and R. Daniel Camerini-Otero (1996) Biophys. J. **70**, A370.

2. Phase behaviors of supported bilayers studied with differential scanning calorimeter.
   Jie Yang (1996) APS March Meeting, New Orleans, March 18-22.

3. AFM studies of lipid structures in supported bilayers.
   Ye Fang and Jie Yang (1996) APS March Meeting, New Orleans, March 18-22.